IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of	:Confirmation No. 7027
Serial No. 10/572,920	: Group Art Unit 1636
Hiroo Iwata et al.	: Attorney Docket No. 2006_0408A
Filed : May 5, 2006	: Examiner KETTER, JAMES S

DECLARATION UNDER 37 CFR 1.132

Honorable Commissioner of Patents and Trademarks

Sir:

I, Hiroo IWATA hereby declare that:

I was born in Wakayama prefecture, Japan, in 1949;

I am a citizen of Japan and a resident of 1-5-8-203, Wakayamadai Shimamoto-cho Mishima-gun, Osaka 618-0024 JAPAN;

I graduated from Department of Polymer Chemistry, Faculty of Engineering, Kyoto University, Japan in 1973.

I received my doctor degree on the study of "Synthesis of Reactive Polymers and Study of Their Polymer-Polymer Reactions" at Kyoto University, Kyoto, Japan, in 1979;

I have worked as a professor of Kyoto University in Japan from 1999 until now and have engaged in a study on Biomedical Engineering;

I am one of the inventors for this application;

I have many reports relating to electroporation using an electrode with a cationic surface. The reports are as follows:

1: Yamauchi F, Koyamatsu Y, Kato K, Iwata H., Layer-by-layer assembly of cationic lipid and plasmid DNA onto gold surface for stent-assisted gene transfer. Biomaterials. 2006 Jun;27(18):3497-504.

2: Yamauchi F, Kato K, Iwata H., Layer-by-layer assembly of poly(ethyleneimine) and plasmid DNA onto transparent indium-tin oxide electrodes for temporally and spatially specific gene transfer. Langmuir. 2005 Aug 30;21(18):8360-7.

3: Yamauchi F, Kato K, Iwata H., Spatially and temporally controlled gene transfer by electroporation into adherent cells on plasmid DNA-loaded electrodes. Nucleic Acids Res. 2004 Dec 21;32(22):e187.

4: Fujimoto H, Kato K, Iwata H., Prolonged durability of electroporation microarrays as a result of addition of saccharides to nucleic acids. Anal Bioanal Chem. 2009 Jan;393(2):607-14.

5: Fujimoto H, Kato K, Iwata H., Electroporation microarray for parallel transfer of small interfering RNA into mammalian cells. Anal Bioanal Chem. 2008 Dec;392(7-8):1309-16.

6: Inoue Y, Fujimoto H, Ogino T, Iwata H., Site-specific gene transfer with high efficiency onto a carbon nanotube-loaded electrode. J R Soc Interface. 2008 Aug 6;5(25):909-18.

7. Koda S, Inoue Y, Iwata H., Gene transfection into adherent cells using electroporation on a dendrimer-modified gold electrode. Langmuir. 2008 Dec 2;24(23):13525-31.

8. Inoue Y, Fujimoto H, Ogino T, Iwata H. Site-specific gene transfer with high efficiency onto a carbon nanotube-loaded

electrode. J R Soc Interface. 2008 Aug 6;5(25):909-18.

9: Njatawidjaja E, Iwata H., Gene delivery to cells on a miniaturized multiwell plate for high-throughput gene function analysis. Anal Bioanal Chem. 2008 Oct;392(3):405-8.

The experiments given below were conducted under my supervision.

Experiment

1. Purpose

The experiment described below was performed to compare nucleic acid introduction-efficiency achieved by two different methods: In the method of our invention, an electric pulse was applied to cells adhering to the surface of a nucleic acid-loaded electrode. In the other method, an electric pulse was applied to cells suspended in a medium using a nucleic acid-loaded electrode.

2. Methods

Example A

A glass plate on which thin layers of chromium (thickness: 1 nm) and gold (thickness: 49 nm) were deposited was used as a main electrode. On this electrode surface, a self-assembled monolayer of 11-mercaptoundecanoic acid was formed. Then, a silicone frame (inner area: 16 mm × 13 mm) with a thickness of 1 mm was attached to the electrode as a spacer and the branched form of poly(ethyleneimine)(PEI) with an average molecular weight of 2,000 was adsorbed from aqueous solution (1%, pH 7.4)

to the electrode surface within the silicone frame.

After washing the main electrode with deionized water, the electrode surface was exposed to a solution of pEGFP-C1 [50 μg/mL in phosphate buffered saline (PBS)] at room temperature for 2 hours to adsorb plasmid onto the main electrode and then extensively washed with PBS. Plasmid pEGFP-C1 is capable of expressing enhanced green fluorescent protein EGFP.

HEK293 cells were seeded onto the plasmid-adsorbed surface of the main electrode at a density of 60,000 cells/cm^2 and cultured in a minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 0.1 mg/ml streptomycin at 37 ℃ for 24 hours to allow cells to adhere onto the plasmid-adsorbed electrode surface.

A counter electrode (a glass plate with a 200 nm-thick gold layer) was placed on the silicone frame attached to the surface of the main electrode, thereby, the distance between the main and counter electrodes was kept at 1 mm by the silicone frame. Then, the lower electrode (the plasmid-loaded main electrode) and the upper electrode (the counter electrode) were connected to a pulse generator (Xcell system manufactured by Bio-Rad Laboratories), and the cells were treated with a single electric pulse at the field strength of 230 V/cm for the duration of 10 msec.

The counter electrode was removed and the cells adhering onto the surface of the main electrode were cultured at 37 ℃ for 3 days.

Then, the main electrode was taken out from the medium, and cells were observed with a microscope. Fluorescent intensity of the cells adhering onto the surface of the main electrode was determined on the fluorescent micrographs acquired at 5 randomly-chosen areas using Image J software (National Institute of Health, U.S.A.). The values of the fluorescent intensity obtained were averaged.

Comparative Example A

A plasmid-adsorbed main electrode was prepared by the same method as described in Example A.

A suspension of HEK293 cells in MEM supplemented with 10% FBS, 100 U/ml penicillin and 0.1 mg/ml streptomycin was added onto the surface of the main electrode within the silicone frame. Thus, a total of 124,800 cells of HEK 293 cells were added to the inner space of the silicone frame. Because the area of the inner space of the silicone frame was 2.08 cm^2, the cell number per cm^2 of the main electrode surface area was 60,000 cells/cm^2 (124,800 cells/2.08cm^2).

Immediately after the addition of cells, a counter electrode was placed on a silicone frame, by which a distance between the main and counter electrodes was kept at 1 mm. Then, a single electric pulse was applied to the cells suspended in the space between the main and counter electrodes at the field strength of 230 V/cm for the duration of 10 msec.

The counter electrode was removed and the cells were

cultured at 37 ℃ for 3 days to adhere onto the surface of the main electrode.

Then, the main electrode was taken out from the medium, and cells were observed with a microscope. Fluorescent intensity of the cells adhering to the surface of the main electrode was determined on the fluorescent micrographs acquired at 5 randomly-chosen areas using Image J software (National Institute of Health, U.S.A.). The values of fluorescent intensity obtained were averaged.

3. Results

Phase contrast and fluorescent micrographs of the cells on the surface of main electrodes are shown in Figure 1 below.

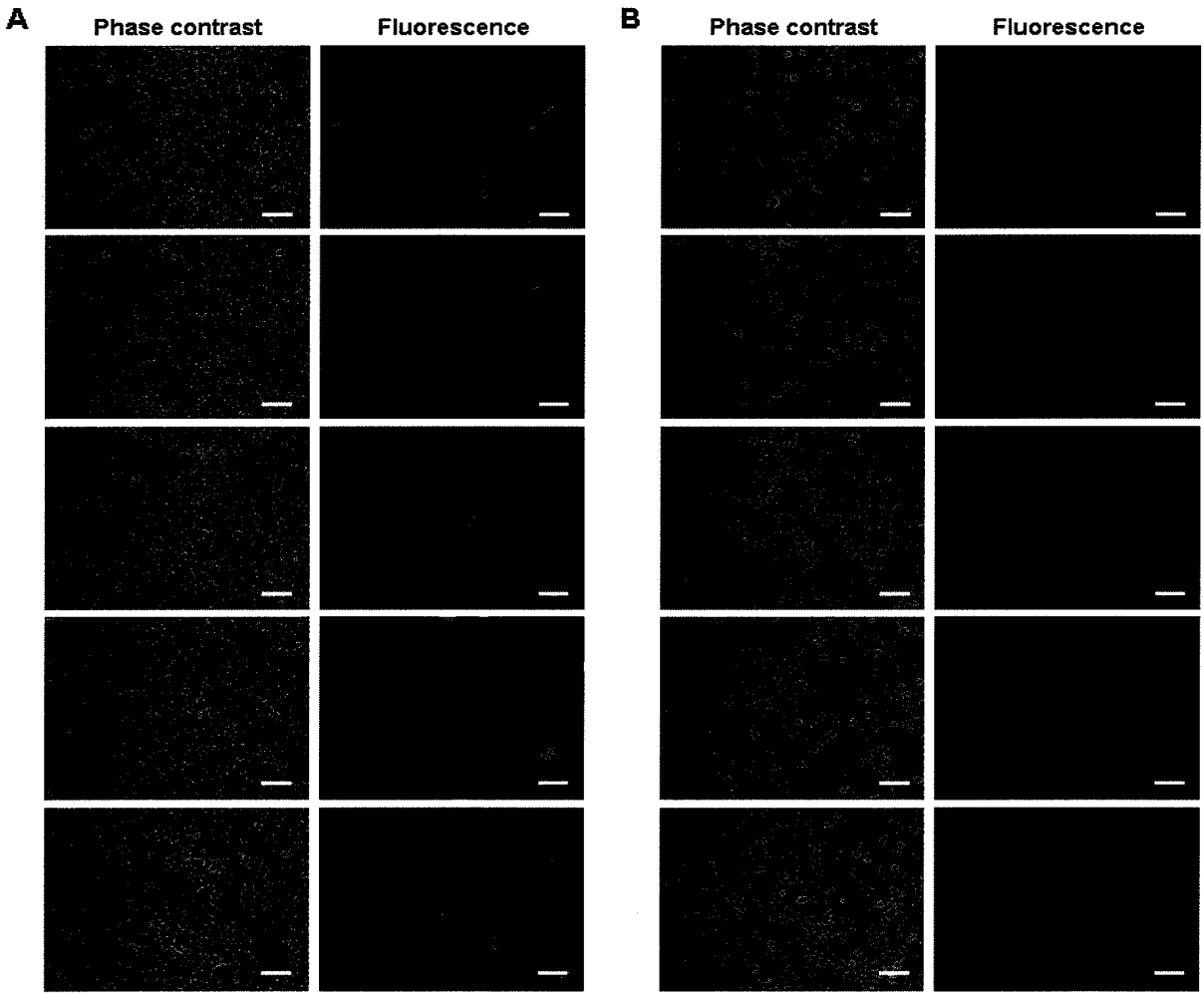

Figure 1. Phase contrast and fluorescent micrographs of cells cultured for 3 days after electric pulsing. An electric pulse was applied to (A) adhering and (B) suspended cells. Micrographs were recorded at 5 randomly-chosen areas. All fluorescent images were acquired with an exposure time of 1.5 sec. Scale bar: 100 μm.

Fluorescent intensities are shown in Table 1 below.

Table 1. Fluorescent intensity of EGFP expressed in transformed cells.

	Fluorescent intensity*
Example A	$8.2 \times 10^6 \pm 3.3 \times 10^6$
Comparative Example A	$1.4 \times 10^5 \pm 0.6 \times 10^5$

*Data are expressed as the mean ± standard deviation (n = 5).

It is not likely that dead cells were kept to adhere to or stay on the surface of the main electrode during cell culture at 37 ℃ for 3 days in a medium. Therefore, obtained fluorescent intensity represents nucleic acid introduction-efficiency mostly for living cells.

As is clear from Figure 1 and Table 1, fluorescent intensity was 59-times higher in transformed cells of Example A than that in transformed cells of Comparative Example A.

As a result, nucleic acid introduction-efficiency of living cells in the case where an electric pulse was applied to cells adhering to the plasmid-loaded electrode was extremely higher than that in the case where an electric pulse was applied to cells suspended in a medium.

It is declared by the undersigned that all statements made herein of undersigned's own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18U.S.C.1001, and that such willful false statements may jeopardize the validity of the above-identified application or any patent issuing thereon.

Date: June 22, 2010

Hiroo Iwata

Hiroo IWATA